Historic, archived document

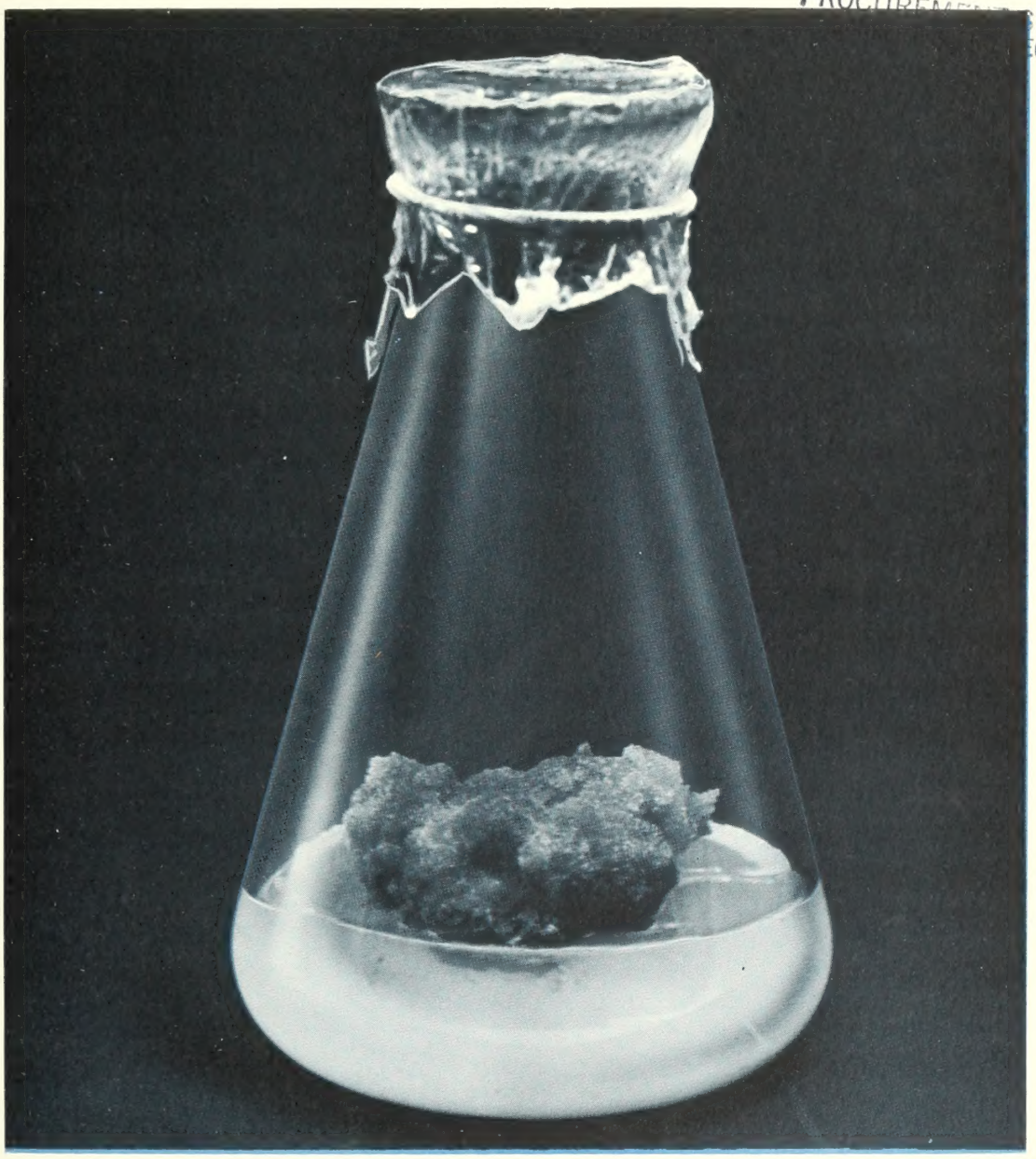
Do not assume content reflects current scientific knowledge, policies, or practices.

64U

PREPARATIVE TECHNIQUES AND TISSUE-SELECTION CRITERIA FOR *IN VITRO* CULTURE OF HEALTHY AND RUST-INFECTED CONIFER TISSUES

U. S. DEPT. OF AGRICULTURE
NATIONAL AGRICULTURAL LIBRARY
RECEIVED
SEP 30 1971
PROCUREMENT SECTION
RECORDS

J. L. Grasham and A. E. Harvey



USDA Forest Service Research Paper INT-82, 1970

INTERMOUNTAIN FOREST AND RANGE
EXPERIMENT STATION
Ogden, Utah 84401

USDA Forest Service
Research Paper INT-82
1970

PREPARATIVE TECHNIQUES AND TISSUE-SELECTION CRITERIA FOR *IN VITRO* CULTURE OF HEALTHY AND RUST-INFECTED CONIFER TISSUES

J. L. Grasham and A. E. Harvey

INTERMOUNTAIN FOREST AND RANGE EXPERIMENT STATION
Forest Service
U.S. Department of Agriculture
Ogden, Utah 84401
Joseph F. Pechanec, Director

THE AUTHORS

J. L. GRASHAM, Biological Research Technician, joined the Intermountain Station staff in January 1966 and is assigned to the Diseases of White Pine Forest Type Research Work Unit at the Forestry Sciences Laboratory, Moscow, Idaho. Previously, he was a Forestry Technician with the Insect and Disease Control Division, Clearwater National Forest, Pierce, Idaho. He attended the University of Arkansas 1957-1958, Connors Junior College 1958-1960, Northeastern State College 1960-1961. Presently, he is working towards a B.S. degree in Agriculture at the University of Idaho, majoring in Plant Sciences.

A. E. HARVEY, Research Plant Pathologist, is presently responsible for research investigations on the biology, host parasite physiology, and biochemistry of the white pine blister rust fungus and for developing methodology of screening and testing chemicals for its control. He is assigned to the Diseases of White Pine Forest Type Research Work Unit at the Forestry Sciences Laboratory, Moscow, Idaho. Dr. Harvey received the B.S. degree in Biology (1960), the M.S. degree in Plant Pathology (1962), and the Ph.D. degree in Plant Pathology (1968) from Washington State University. He joined the Intermountain Station staff in 1965.

CONTENTS

	Page
INTRODUCTION	1
TECHNIQUES	1
Preparation of Healthy Conifer Tissues	1
Preparation of Blister Rust-Infected Western White Pine Tissues	3
DISCUSSION	4
CONCLUSIONS	5
LITERATURE CITED	5

The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others which may be suitable.

ABSTRACT

Selection of young stem sections free of external injury and the reduction of physical and/or chemical damage to tissues during their preparation, sterilization, and excision were the most important factors governing survival and growth of healthy and diseased conifer tissues *in vitro*. Most (75-98 percent) primary explants from properly selected stems, prepared as described, survived and produced subsequent growth on suitable media. Proliferation of the rust (*Cronartium ribicola* J. C. Fisch. ex Rabenh.) occurred in 75 percent of the surviving blister rust-infected primary explants from western white pine (*Pinus monticola* Dougl.).

INTRODUCTION

The tissue culture technique has long been used to study the physiology of specific plants, plant organs, and tissues. By employing this technique, one can control the environment, the substrate, and (if he uses clonal stock) the genetic composition of the tissues under observation. Thus, the effects of variables on such studies can be minimized or eliminated.

It is recognized that members of the order Coniferales are difficult to propagate vegetatively *in vitro* (Loewenberg and Skoog 1952; Reinert 1962). Researchers have reported excessive tissue damage and/or secondary contamination during the primary explanting stage (Gautheret 1959; Koenigs 1968). In the authors' opinion, failures to culture conifer tissues have been due to improper preparative techniques and tissue-selection criteria. Our success with several conifer species (Harvey 1967; Harvey and Grasham 1969a) and with rust-infected western white pine, *Pinus monticola* Dougl., (Harvey and Grasham 1969b) prompted us to prepare this paper detailing improved techniques.

TECHNIQUES

To be successful, our techniques require timing as well as treatment; so we have outlined them in order, from sample selection through explant incubation.

Preparation of Healthy Conifer Tissues

Tissue-selection criteria.--Optimum tissue culture proliferation was obtained by using primary explants from current-year stems of either vigorous nursery stock or field-grown trees. Satisfactory results were also obtained by using older material, but callus development and growth were not as rapid. Callus development was delayed when we cultured material collected in Idaho during the months of December, January, and February.

Contamination was kept minimal by using stem sections that had a smooth bark, free of insect or mechanical damage (figs. 1 and 2). Except for the fungi and bacteria indigenous to the cortex, subepidermal bark tissues were relatively free of contamination.

Cultures were prepared the day that material was collected. Although overnight refrigeration of material before preparation was acceptable, further delay allowed tissue oxidation and contamination buildup, both of which are severely detrimental to *in vitro* tissue growth.

Pre-sterilization preparation.--Needles were clipped flush with the bark surface before the stems were sterilized. Care was taken to avoid damage to bark tissues during this operation. Tears permitted the sterilizing fluids used during subsequent operations to penetrate and injure cortical tissues. Tissue damaged by excessive penetration of sterilizing fluids proliferated poorly *in vitro*.

Stems from which needles had been removed were sectioned into 2-3-cm. lengths by means of a high-speed, fine-bladed jigsaw (fig. 3). The fraying action of the reciprocating blade tended to seal the open ends, which prevented excessive penetration by sterilizing fluids. End pieces were extensively damaged at the time of collection and were always discarded.

Cut sections were washed for 1 hour under rapidly running tapwater to free them of dirt and other foreign matter. In our laboratory, a longer washing period resulted in contamination by bacteria commonly associated with the water supply. Water-saturated stem sections were not satisfactory to tissue culture for this reason.

Sterilization.--After being washed, the sections were gently shaken in 200-500 mℓ. of a 5.25% sodium hypochloride (NaOCl) solution contained in a 1-liter, screw-top flask. No more than 25 sections were treated at one time in order to minimize the total contaminant load and to assure proper sterilization. If stem sections were properly chosen, they required only 1 to 3 minutes of surface sterilization. Since the cortex is well-protected by the unbroken epidermis of current-year stems, it was relatively free of contamination and only surface sterilization was necessary. An alternate treatment, the use of a 3% hydrogen-peroxide (H_2O_2) solution for 24 hours, successfully controlled contamination of conifer species *(Pinus nigra, P. ponderosa, Larix occidentalis)* easily damaged by the NaOCl solution (Harvey and Grasham 1969a). Whenever cortical tissues had low levels of surface contamination, decreasing sterilization periods resulted in better tissue culture development, although for most tissues more time was needed to control contamination. An increase in sterilization delayed callus development. Immediately following sterilization with either NaOCl or H_2O_2, stem sections were rinsed three to five times in cool, sterile distilled water to remove all traces of the sterilant. (Complete removal of the sterilizing agent is necessary for normal callus development.)

Excision of primary explants.--Immediately after sterilization, cortical tissues were aseptically excised from the stem sections and transferred to the desired medium. These tissues constituted the primary explants.

During the excision process, all tools were kept cool (room temperature), extremely sharp, and free of the sterilizing agent. Razor blades and scalpels were changed frequently to prevent dull or damaged tools from tearing or bruising the tissue. Bruising or tearing of the cortical tissues during excision caused discoloration and abnormal development of the callus tissue. Similar effects were observed when tissues came in contact with hot instruments or with a sterilizing agent.

Each stem section was separately removed from the flask in a sterile room and placed on a sterile surface. Each section was held gently but firmly by sterile forceps. A sterile blade was then used to make a longitudinal slit through the bark and cambium (fig. 4). The Bard Parker surgical blade No. 11 was satisfactory. Forceps were then used to rotate the section while the cuticle and epidermis were surgically removed in thin, longitudinal strips (fig. 5). (Several fungi are associated with these tissues; so complete removal of these layers is essential.) Extreme care was taken not to bruise cortical tissues during this process. Cortical tissues were then undercut in the cambial zone (fig. 6) and carefully lifted from the woody cylinder (fig. 7). The wood was discarded. The damaged ends of the cortical tissues were trimmed, and the remaining tissues cut into 100-150-mm.^{2} rectangular pieces. These pieces then were placed, cambium side down, on culture media. Optimum tissue culture growth and callus proliferation were obtained when large (100-150 mm.^{2}) primary explants were used. All working surfaces and tools were resterilized with alcohol, flame-dried, and air-cooled before the next section was prepared.

Incubation vessels.--Standard flare-mouth culture tubes (25 X 100 mm.) covered with a single layer of plastic food wrap were used successfully as incubation vessels. The tubes contained 15 mℓ. of medium and proved to be satisfactory for tests of 90 days' duration. Similarly covered, 125-mℓ., wide-mouthed Erlenmeyer flasks, containing 50 mℓ. of medium, produced superior growth when cultures were maintained more than 90 days.

The plastic film closures replaced Dispo foam plugs that were used during preparation of the media and as closures until all primary explants had been placed on a culture medium.

Closures were prepared by pressing large sheets of plastic food wrap on an alcohol-dampened surface for sterilization. Sheets then were cut in place with a razor blade into 6-cm. squares and placed, sterile side down, over the mouth of the vessel. A tight-fitting rubber band secured the closure.

Plastic film closures were superior to other types of closures tested. Tissue cultures grown in tubes that had cork or screw-cap tops showed erratic growth. Bacteriological stoppers or cotton plugs permitted media to dry too rapidly for our needs.

Incubation environment.--Explants were incubated at 20° C. ±2° with light for 16 hours and then at 5° C. ±2° without light for 8 hours (Harvey and Grasham 1969b). Cool, white fluorescent light at an intensity of 400 ft.-c. was satisfactory. Tissue cultures grown in darkness or semidarkness lacked vigor compared to those grown under light. Optimum results were obtained by beginning incubation of the explants at the start of the cool, dark period of the incubation cycle.

Medium.--Good proliferation was obtained with many conifer species (Harvey 1967; Harvey and Grasham 1969a) by using a simple medium consisting of the essential auxins, indoleacetic acid (IAA), napthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D), at concentrations between .01 and 5 mg./ℓ.

Addition to the medium of certain amino acids, vitamins, and other growth factors further enhanced growth and development of many species (Harvey and Grasham 1969a). Tissue cultures benefited most when such compounds were used in microgram quantities (10, 100, 1,000 µg./ℓ.).

All *heat-sensitive organic* compounds were sterilized by *filtration* and added to the autoclaved, basal salt medium (Harvey and Grasham 1969a) after it had cooled to 50° C.

The pH of the medium played an important role in the initial growth of the cambium layer. Our work indicated that a pH range of 4.0 to 6.0 was suitable for the initial growth of most cambium explants (Harvey and Grasham 1969a).

Preparation of Blister Rust-Infected Western White Pine Tissues

Save for minor modifications, these same techniques were used for the *in vitro* culture of rust-infected western white pine (Harvey and Grasham 1969b). The proper selection of stem sections is critical to the culture of infected material. Material chosen must be 2 to 3 years older than that used in healthy tissue studies to assure rust infection and ramification. Nonfruiting rust cankers from 2- to 3-year-old growth segments of 6- to 10-year-old seedlings have been the best source of infected material.

After the needles were removed in the laboratory, the stems were sectioned as previously described. Only the outer edge of the infection was used. Stem sections were cut so that they were one-eighth infected and seven-eighths healthy tissue (fig. 3).

A sterilization period of 5-10 minutes using 5.25% NaOCl solution was necessary to adequately control contamination. Again, all traces of the sterilant were removed by rinsing the sections three to five times in cool, sterile distilled water.

Bark sections were excised according to the procedure outlined for healthy tissue, except that cortical tissues were divided into rectangular pieces (100-150 mm.^{2}). One-eighth of each rectangle was infected tissue, seven-eighths of each piece, healthy tissue (fig. 7).

Seventy-five to 98 percent survival and subsequent growth of healthy and blister rust-infected white pine (Harvey 1967; Harvey and Grasham 1969b) and a minimum of 75 percent survival and growth of other species (Harvey and Grasham 1969a) were obtained on artificial media by following these techniques.

DISCUSSION

The delay in tissue development experienced during the winter months appeared to be related to dormancy. The dormancy requirement apparently had not been satisfied. As a result, the physiological condition of this tissue was less receptive to auxin-induced growth and an adjustment period (not required by active tissue) was necessary.

The reduced surface oxidation and subsequent increase in growth noted when cultures were started at the beginning of the cool period were apparently related to the effects of temperature on the rate of chemical oxidation. The lower temperature may also have permitted the tissue to neutralize the effects of oxidation.

The use of large primary explants assures: (1) substantial food reserves necessary to maintain the explants until proliferation begins; and (2) reduction of the injured:uninjured tissue ratio to minimize the effects of damages incurred during tissue preparation.

The one-eighth infected to seven-eighths healthy tissue ratio used to culture rust-infected tissue allowed for good host development and adequate inoculum potential for rust proliferation. This ratio is important to successful culture of this host-parasite system and produces optimum results.

The increase of growth experienced when plastic vessel closures were used was apparently related to: (1) increased light transmission; (2) the conservation of moisture in the vessel; and (3) an adequate exchange of gases. The requirement for a balanced system of gaseous exchange and moisture conservation was effectively demonstrated when closures permitted either excessive moisture loss (cotton) or no gas exchange (screw caps or corks).

Since all other cultural conditions were identical, the increased growth obtained in 125-mℓ. flasks over that in 25 X 100 mm. tubes was due to the amount of medium and/or gas volume available to the tissues.

In our experience, failures of primary explants to proliferate into callus tissues were usually related to one or more of the following procedural errors:

1.--Improper selection of plant materials;
2.--Delay in excision of tissues after collection of materials;
3.--Improper length of time for sterilization;
4.--Inadequate removal of a sterilizing agent;
5.--Incomplete removal of the epidermis;
6.--Damage to cortical tissues from hot or dull excision blades;
7.--Bruising or tearing of the excised tissues;
8.--Delay in transferring excised tissues to a culture medium;
9.--Improper incubation conditions;
10.--Incorrect selection or concentration of auxin; and
11.--Improper pH of the medium.

CONCLUSIONS

The techniques described herein increase the efficiency of culturing a wide variety of conifer tissues. These techniques may also be beneficial to the *in vitro* establishment of many obligate parasites of cultured species. These techniques should not only improve results obtained in culturing conifer species, but may be applicable to the culture of other members of the plant kingdom.

LITERATURE CITED

Gautheret, R. J.
1959. La culture des tissus végétaux: techniques et réalisations. Masson et Cie, Paris. 863 p.

Harvey, A. E.
1967. Tissue culture of *Pinus monticola* on a chemically defined medium. Can. J. Bot. 45: 1783-1787.

_____, and J. L. Grasham.
1969a. Procedures and media for obtaining tissue cultures of 12 conifer species. Can. J. Bot. 47: 547-549.

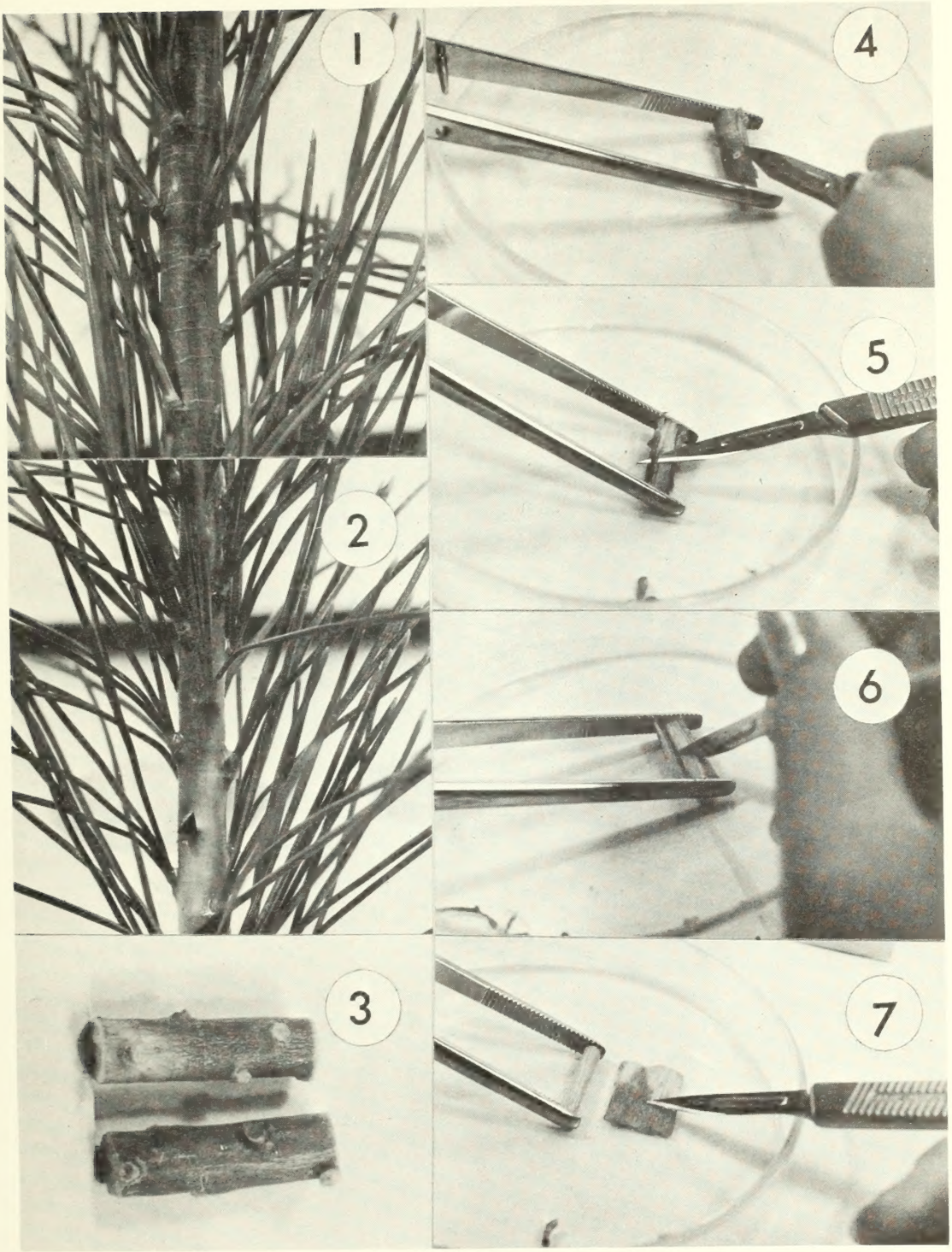
_____, and J. L. Grasham.
1969b. Growth of the rust fungus *Cronartium ribicola* in tissue cultures of *Pinus monticola*. Can. J. Bot. 47: 663-666.

Koenigs, J. W.
1968. Culturing the white pine blister rust fungus in callus of western white pine. Phytopathology 58: 46-48.

Loewenberg, J., and F. Skoog.
1952. Pine tissue cultures. Physiol. Plant. 5: 33-36.

Reinert, J.
1962. Morphogenesis in plant tissue cultures. Endeavour 21: 85-90.

Figures 1-7. The selection and preparation of healthy and parasitized conifer explants for tissue culture. Figures 1 and 2, the type of stems found most suitable for tissue culture explants of healthy and infected western white pine and other conifer species. Arrow denotes the discolored edge of the rust-infected zone in figure 2 (2.5X). Figure 3, 2- to 3-cm. sections of healthy (lower) and rust-infected (upper) current-year stems of western white pine prepared for sterilization (2X). Figures 4, 5, 6, and 7, stepwise removal of an infected cambium strip from western white pine, after surface sterilization (3/4X).


1
2
3
4
5
6
7

Headquarters for the Intermountain Forest and Range Experiment Station are in Ogden, Utah. Field Research Work Units are maintained in:

Boise, Idaho
Bozeman, Montana (in cooperation with Montana State University)
Logan, Utah (in cooperation with Utah State University)
Missoula, Montana (in cooperation with University of Montana)
Moscow, Idaho (in cooperation with the University of Idaho)
Provo, Utah (in cooperation with Brigham Young University)